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ORIGINAL ARTICLE

Three New Ranidae Mitogenomes and the Evolution of Mitochondrial Gene Rearrangements among Ranidae Species

Jiandong YANG[1,#,*], Jiaojiao YU[1,#], Jiabin LIU[2], Ming ZHOU[1], Biao LI[1] and Bo OUYANG[1]

[1] *College of Animal Science and Technology*, *Sichuan Agricultural University*, *Chengdu* 611130, *Sichuan*, *China*
[2] *Sichuan Key Laboratory of Conservation Biology for Endangered Wildlife*, *Chengdu Research Base of Giant Panda Breeding*, *Chengdu* 610081, *Sichuan*, *China*

Abstract Various types of gene rearrangements have been discovered in the mitogenoes of the frog family Ranidae. In this study, we determined the complete mitogenome sequence of three *Rana* frogs. By combining the available mitogenomic data sets from GenBank, we evaluated the phylogenetic relationships of Ranidae at the mitogenome level and analyzed mitogenome rearrangement cases within Ranidae. The three frogs shared an identical mitogenome organization that was extremely similar to the typical Neobatrachian-type arrangement. Except for the genus *Babina*, the monophyly of each genus was well supported. The genus *Amnirana* occupied the most basal position among the Ranidae. The [*Lithobates* + *Rana*] was the closest sister group of *Odorrana*. The diversity of mitochondrial gene arrangements in ranid species was unexpectedly high, with 47 mitogenomes from 40 ranids being classified into 10 different gene rearrangement types. Some taxa owned their unique gene rearrangement characteristics, which had significant implication for their phylogeny analysis. All rearrangement events discovered in the Ranidae mitogenomes can be explained by the duplication and random loss model.

Keywords mitochondrial genomes, gene rearrangement, molecular phylogeny, Ranidae

1. Introduction

Previous studies had revealed that the gene organization in vertebrate mitogenomes is conserved and that the mitochondrial D-loop region and the 37 genes were arranged in same manner among vertebrates (Anderson *et al.*, 1981; Roe *et al.*, 1985; Tzeng *et al.*, 1992; Zardoya *et al.*, 1995). However, numerous gene rearrangements in the mitogenome can independently evolve (Alam *et al.*, 2010; Chen *et al.*, 2011; Desjardins and Morais, 1990; Kurabayashi *et al.*, 2006, 2008, 2010; Liu *et al.*, 2005; Mindell *et al.*, 1998; Moritz and Brown, 1987; Sano *et al.*, 2005; Su *et al.*, 2007; Zhang *et al.*, 2013). Gene rearrangements involve duplications, losses, translocation, inversion, and/or shuffling of the D-loop region (also known as the control region), the replication origin of the light strand (O_L) and the codon genes (including rRNA genes, tRNA genes and protein-coding genes). Although distinct mitogenome structural features have been reported for some amphibians, most amphibians (including caecilians, salamanders, archaeobatrachians, and mesobatrachians) generally conform to the typical Vertebrate-type mitochondrial gene arrangement (Liu *et al.*, 2016; Mueller and Boore, 2005; Pabijan *et al.*, 2008; San Mauro *et al.*, 2004, 2006, 2014; Xia *et al.*, 2010; Zhang *et al.*, 2008; Zhang and Wake, 2009). Surprisingly, the gene arrangements in the neobatrachian group are especially diverse and complex, and notably, their four tRNA genes (*LTPF-trn*) are commonly rearranged, which is distinguishable from the vertebrate ancestral gene order (Kurabayashi *et al.*, 2010; Sumida *et al.*, 2001; Xia *et al.*, 2014).

The vertebrate mitochondrial rearrangements appear to be unique, random, generally rare events that are exceptionally unlikely to arise independently

[#] Both authors contributed equally to this paper.
[*] Corresponding authors: Dr. Jiandong YANG, from Sichuan Agricultural University, Sichuan, China, with his research focusing on taxonomy, evolution and molecular ecology of amphibians.
E-mail: yangjd@sicau.edu.cn
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in independent evolutionary lineages (Boore and Brown, 1998; Liu and Huang, 2010; Xia *et al.*, 2010, 2014), although a few convergent or parallel gene rearrangements have been observed in vertebrate mtDNAs (Morrison *et al.*, 2002; Wei *et al.*, 2014). The exceptional mitochondrial gene rearrangement has been thought to have significant implication for animal phylogenetic analysis and is considered a powerful phylogenetic marker also applicable to explore phylogenetic relationships among various groups at different taxonomic levels (Boore and Brown, 1998; Macey *et al.*, 1997; San Mauro *et al.*, 2004, 2014; Wei *et al.*, 2014; Xia *et al.*, 2010; Xue *et al.*, 2016; Zhang *et al.*, 2008, 2009, 2013). For example, *Odorrana tormota*, a species famous for its ultrasonic communication, was previously regarded as a member of *Amolops* (Frost, 2017). However, this frog shares the same mitochondrial gene arrangement (the *trnH* was translocated to D-loop downstream, forming a *HLTPF-trn* cluster) with most *Odorrana* frogs, not with the *Amolops* frogs (conventional *LTPF-trn* cluster) (Su *et al.*, 2007).

The family Ranidae, also known as ranid frogs, is one of the most species-rich and fascinating groups of vertebrates (Che *et al.*, 2007; Li *et al.*, 2014; Frost, 2017). Ranidae represents one of the main components of Neobatrachia and contains approximately 380 described species, belonging to 23–24 genera (AmphibiaWeb, 2017; Frost, 2017). A total of 31 complete and 12 near-complete ranid mitochondrial genomes have been submitted to GenBank, and many novel gene rearrangement types have also been discovered (e.g. Li *et al.*, 2014, 2016a, b; Kurabayashi *et al.*, 2010; Su *et al.*, 2007). Kurabayashi *et al.* (2010) reported the partial or complete mtDNAs of 10 ranids and found most mitogenomes were different from the typical Neobatrachian-type gene arrangement. The diversity of mitochondrial gene arrangements in ranid species is unexpected high (Kurabayashi *et al.*, 2010).

Here, we decode the mitochondrial genomes of three ranid frogs, conduct comparative genome analysis with all available Ranidae mitogenome sequences submitted to GenBank, and perform the phylogenetic analysis among Ranidae species. Our aim was to conduct an in-depth investigation, including examining the phylogenetic relationships, redescribing the novel mitogenome structures, analyzing exhaustively the genome reorganization types, and inferring the possible mechanisms and evolutionary pathways of gene rearrangements as well as its systematic implication among ranid frogs. Our study helps to understand mitogenome evolution and phylogenetic relationships of Ranidae species.

2. Materials and Methods

2.1. Specimen collection, DNA extraction, and PCR amplification Specimens of *Rana kukunoris*, *R. chaochiaoensis* and *R. omeimontis* were obtained from Zoige County (33.57066° N, 102.96348° E, 3446 m a.s.l.), Shimian County (29.02461° N, 102.38626° E, 2 085 m a.s.l.), and Yucheng District (29.97900° N, 102.98117° E, 618 m a.s.l.) in Sichuan Province, China, respectively, and stored at −80°C. A TaKaRa MiniBEST Universal Genomic DNA Extraction Kit Ver.5.0 (Takara, Dalian, China) was used to extract total genomic DNA from a frozen tissue sample of the thigh muscle according to the detailed manufacturer's protocol. Primer sets used to amplify the entire mitogenomes of the three *Rana* species are shown in Table S1.

2.2. Sequence assembly and annotation The overlapping sequence fragments were assembled by the program Seqmen (DNAstar, Madison, WI, USA). The annotations of rRNA genes (rRNAs), tRNAs, protein coding genes (PCGs) and D-loop region and the definitions of their respective gene boundaries were performed by the MitoAnnotator service (http://mitofish.aori.u-tokyo.ac.jp/annotation/input.html). The ARWEN program (http://mbio-serv2.mbioekol.lu.se/ARWEN/) was also utilized to infer the tRNAs via their proposed cloverleaf secondary structure and anticodon sequences. All annotation results were verified via alignment with homologous regions from other reported *Rana* mitochondrial genomes. Finally, the mitochondrial genetic diagrams were generated by the OGDRAW program (http://ogdraw.mpimp-golm.mpg.de).

2.3. Data collection We downloaded 32 complete and 12 partial Ranidae mitochondrial genomes from GenBank (Table 1). Eight non-Ranidae mitogenomes were used as out-groups in the phylogenetic analysis. The taxonomic names of all species were based on 'Amphibian Species of the World 6.0' (Frost, 2017). There were many errors in some mitogenome annotations previously submitted to GenBank, and these mitogenome sequences should be re-annotated in systematic or comparative research (Cameron, 2014). In order to avoid interference caused by these errors in our subsequent analysis, we reanalyzed all sequences using the online services MitoAnnotator and ARWEN. The important corrections were listed in Figure S1.

2.4. Genome rearrangement analysis We compared

and analyzed re-annotated mitogenomes, together with the three new *Rana* frog data, with respect to mitogenome gene order (Chen *et al.*, 2011). The definition of mitogenome organization types is based on the comparative results. To clarify, if the gene arrangements of the new mitogenome deviate from the typical Vertebrate-type gene arrangement (Type A) and the typical Neobatrachian-type gene arrangement (Type B), we will divide it into a new type (Figure 1). The long intergenic spacer frequently found in the closely related species and the pseudogene are also taken into account. If we cannot determine that the long intergenic spacer (more than 20 bp in size) frequently found in the closely related species is a pseudogene via homologous sequence alignments, for convenience, we will temporarily call it as "gap" in this study.

2.5. Phylogenetic tree analysis Firstly, all termination codons of 13 PCGs nucleotide sequences were manually deleted. Then, the remaining fragments of each PCG were separately aligned based on their translated amino acid sequences by Muscle implemented in MEGA6.06 (Tamura *et al.*, 2013), and the two rRNAs sequences were separately aligned by ClustalX2 (Larkin *et al.*, 2007). Subsequently, all ambiguous alignment regions were trimmed by the Gblocks Server (http://molevol.cmima.csic.es/castresana/Gblocks_server.html), the type of sequence was set to Codons (for PCGs) or DNA (for rRNAs) and all options for a less stringent selection were selected. Finally, the 15 trimmed alignments were concatenated into a single dataset to infer the phylogenetic relationships of Ranidae. For the concatenated sequence matrix, two phylogenetic trees were constructed using both Bayesian inference (BI) and maximum likelihood

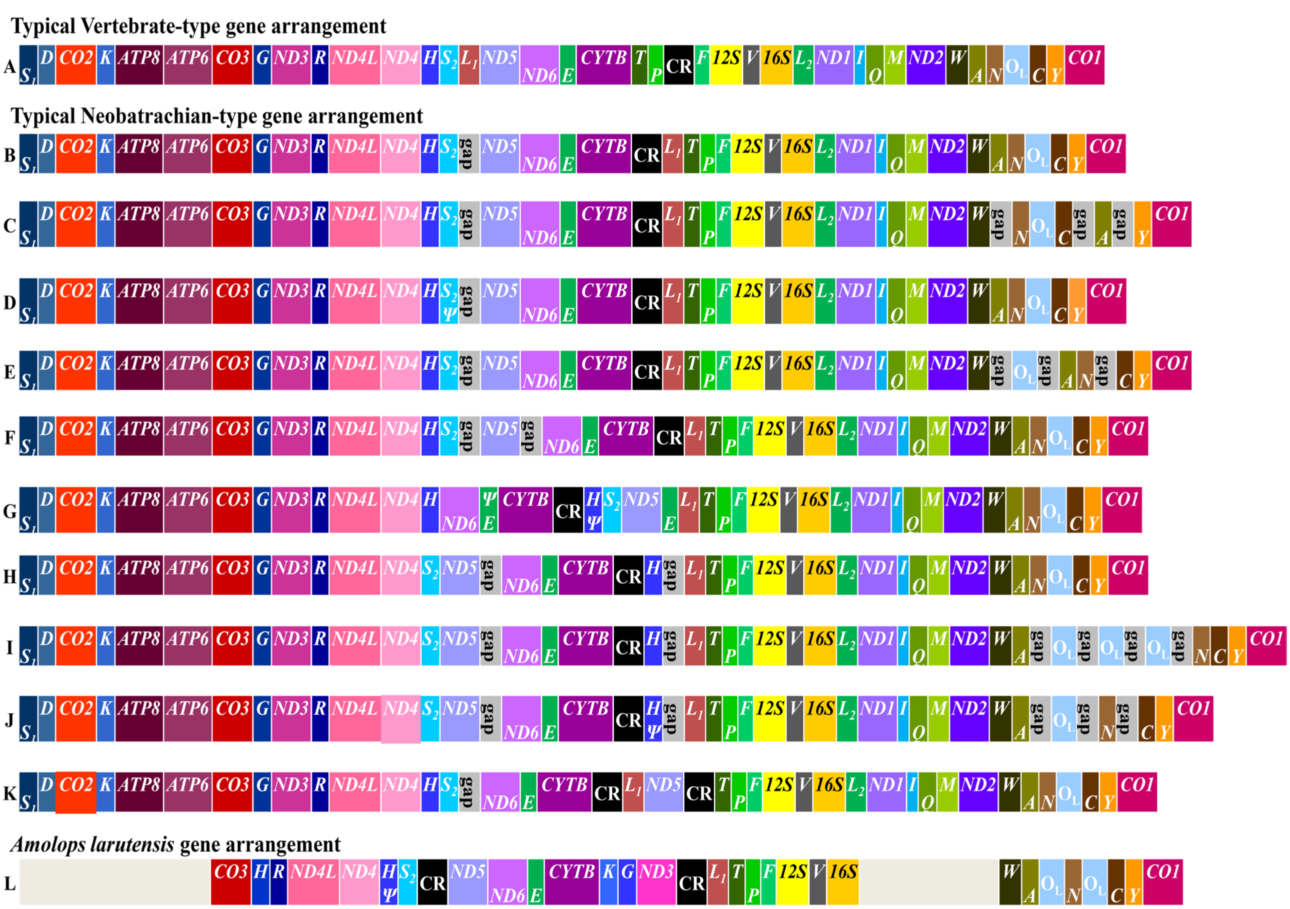


Figure 1 Mitochondrial genomic organizations of Ranidae frogs. Each tRNA gene is represented by the standard one-letter amino acid code, and $S_1 = trnS^{UCN}$, $S_2 = trnS^{AGY}$, $L_1 = trnL^{CUN}$, $L_2 = trnL^{UUR}$. Other genes are abbreviated as follows: *12S* and *16S*, 12S and 16S ribosomal RNA; *ATP6* and *ATP8*, adenine triphosphatase subunits 6 and 8; *CO1–3*, cytochrome c oxidase subunits 1–3; *CYTB*, cytochrome b; *ND1–6* and *4L*, NADH dehydrogenase subunits 1–6 and 4L. O_L, CR, Ψ, and gap denote replication origin of light strand, D-loop region, pseudogene, and intengenic spacer region, respectively. Genes encoded by the heavy and light strand are denoted at the top and bottom of each gene rectangle box, respectively. The sizes of the boxes do not reflect actual gene length.

(ML) approaches. The ML analysis was conducted by PhyML3.1 (Guindon *et al.*, 2010) under the GTR + I + G evolutionary model determined by jModelTest2.1.5 (Darriba *et al.*, 2012), with 100 replicates for the non-parametric bootstrap analysis. The BI analysis was performed by MrBayes3.2.2 (Ronquist and Huelsenbeck, 2003). For the BI analysis, we firstly partitioned the data into 15 partitions by gene, and then used jModelTest2.1.5 to select the best-fit model of nucleotide substitution for each partition with the Bayesian Information Criterion, which was preferred for model selection (Luo *et al.*, 2010). We performed two independent runs for 5 000 000 generations, sampled every 1 000 generations, conservatively discarded the first 25% of generations as burn-in, and visualized the majority-rule (>50%) consensus trees using FigTree1.4.2 (http://tree.bio.ed.ac.uk/software/figtree/).

3. Results

3.1. Mitogenome Characterization and analysis of three new *Rana* mitogenomes

3.1.1. Genome organization The complete nucleotide sequences of the *R. chaochiaoensis*, *R. kukunoris* and *R. omeimontis* mitogenomes have been determined successfully in this study and submitted to the GenBank database under accession numbers KU246048–KU246050 (Table 1). All three mitogenomes were circular, consisting of two rRNAs, 13 PCGs, 22 tRNAs and four intergenic spacer regions (Table S2; Figure S2). The largest intergenic spacer region was located between *CYTB* and $trnL^{CUN}$, which was the typical position of D-loop region. We determined the smaller one located in the *WANCY-trn* cluster as O_L region based on its typical stem-loop structure and the surrounding 5'-GCCGG-3' motif (on the light strand). The remaining two gaps were discovered at the two flanks of *ND5* gene (Figure S2). All three mitogenomes retained the identical genomic organization (Figure 1; Figure S2), and they were 18 591 bp, 18 863 bp, and 19 934 bp in size, respectively (Table 1). The overall base composition of the light strand was 28.85%–29.51% for T, 28.04%–28.45% for C, 27.46%–27.88% for A and 14.56%–15.06% for G with an A + T bias (56.49%–57.39%).

3.1.2. Ribosomal RNA and Protein-Coding genes The *12S* and *16S rRNA* of three mitogenomes were located between *trnF* and $trnL^{UUR}$ and separated by *trnV*. The size of *12S* and *16S rRNA* were 931 bp and 1582 bp for *R. omeimontis*, 930 bp and 1576 bp for *R. chaochiaoensis*, and 929 bp and 1 576 bp for *R. kukunoris*, respectively (Table S2). The overall base composition of two rRNAs were shown as A > C > T > G.

All mitochondrial genomes shared a set of 13 PCGs, including *ND1–6*, *ND4L*, *CO1–3*, *ATP8*, *ATP6* and *CYTB*, and only *ND6* was encoded on the L-strain (Table S2; Figure S2). Most PCGs began with the typical ATG codon, excepting *CO1*, *ATP6* and *ND4L* initiated with GTG, and *ND1* started at ATC (for *R. omeimontis*) and GTG (*R. chaochiaoensis* and *R. kukunoris*). Six PCGs harbored the traditional complete termination codons TAA (*ATP8*, *ND4L* and *CYTB*), AGA (*ND5* and *ND6*) and AGG (*CO1*), whereas the remaining seven PCGs used T (Table S2).

3.1.3. Transfer RNA genes Excluding the $trnS^{AGY}$ gene, the inferred secondary structures of the other 21 tRNAs of the three mitogenomes conform to the common structural features of mitochondrial tRNAs (Table S2; Figure 1). The base mutations of tRNAs among three mitogenomes existed in the stems and the loops structure.

3.2. Molecular phylogenetic analysis The final concatenated mtDNA sequence matrix for 48 species was 13 737 bp in size, including 8 777 variable sites of which 974 were singleton sites. Two phylogenetic reconstruction methods (ML and BI) yielded identical tree topologies based on 13 PCGs and two rRNAs, and they favored the following clades and/or relationships of Ranidae (Figure 2): (1) the most basal position of the genus *Amnirana*; (2) the secondary basal position of the genus *Glandirana*; (3) the clade of *Pelophylax* + *Amolops*; (4) the paraphyly of *Babina* interweaved with *Sylvirana*; (5) the clade of *Odorrana*; (6) the monophyly of *Lithobates* and *Rana*; (7) the clade *Babina* + (*Odorrana* + (*Lithobates* + *Rana*)). Within the lineage Ranidae, clade 7 formed the sister taxon to clade 3, but no sufficient statistical support existed for this relationship (BP = 41, BPP = 0.90).

3.3. Ranidae gene rearrangement analysis According to our comparison of genome organization, we summarized 10 different gene arrangements (Figure 1; Table 2). All rearrangements occurred in both the *ND4–trnT* and the *trnW–CO1* regions (Figure 1; Figure 3).

Our results showed that Type B (also termed as the typical Neobatrachian-type arrangement) was the most common type in ranid (or neobatrachian) mitogenomes. All *Pelophylax* frogs and another two *Amolops* frogs, *A. ricketti* and *A. wuyiensis* (namely the *A. ricketti* species group), expressed the Type B. Additionally, Type B was the most basic type, and another nine novel types (from Type C to Type K) were derived from it via diverse

rearrangement pathways.

Type C was only discovered in *Amnirana albolabris* (Figure 2; Table 2). In this type, the positions between *trnA* and *trnN*-O_L-*trnC* were exchanged accompanied with the insertions of some non-coding regions and finally yielding the novel *trnW*-gap-*trnN*-O_L-*trnC*-gap-*trnA*-gap-*trnY* order (Figure 3). Type D was unique to the *Glandirana* frogs, which was characterized by the $trnS^{AGY}$ pseudogene next to *trnH* (Figure 2; Table 2). Type E was shared by *Amolops mantzorum* species group, which was different from the Type B possessed by the *A. ricketti* species group in terms of location of the O_L structure (Figure 2; Table 2). The O_L was translocated from the downstream to the upstream position of the *trnA*-*trnN*, and then several non-coding regions were inserted into this block, yielding the distinctive *trnW*-gap-O_L-gap-*trnA*-*trnN*-gap-*trnC*-*trnY* order (Figure 3).

Type F was the most common type (32.50%) in ranid mitogenomes so far (Table 3). Type F appeared in most of *Rana* (including our three species), all *Lithobates*, several *Babina* and one *Sylvirana* frogs (Figure 2; Table 2). Type G was shared by the two *Babina* frogs (Figure 2; Table 2). A large number of gene rearrangements were found in Type G. The variation of gene rearrangement in *Odorrana*

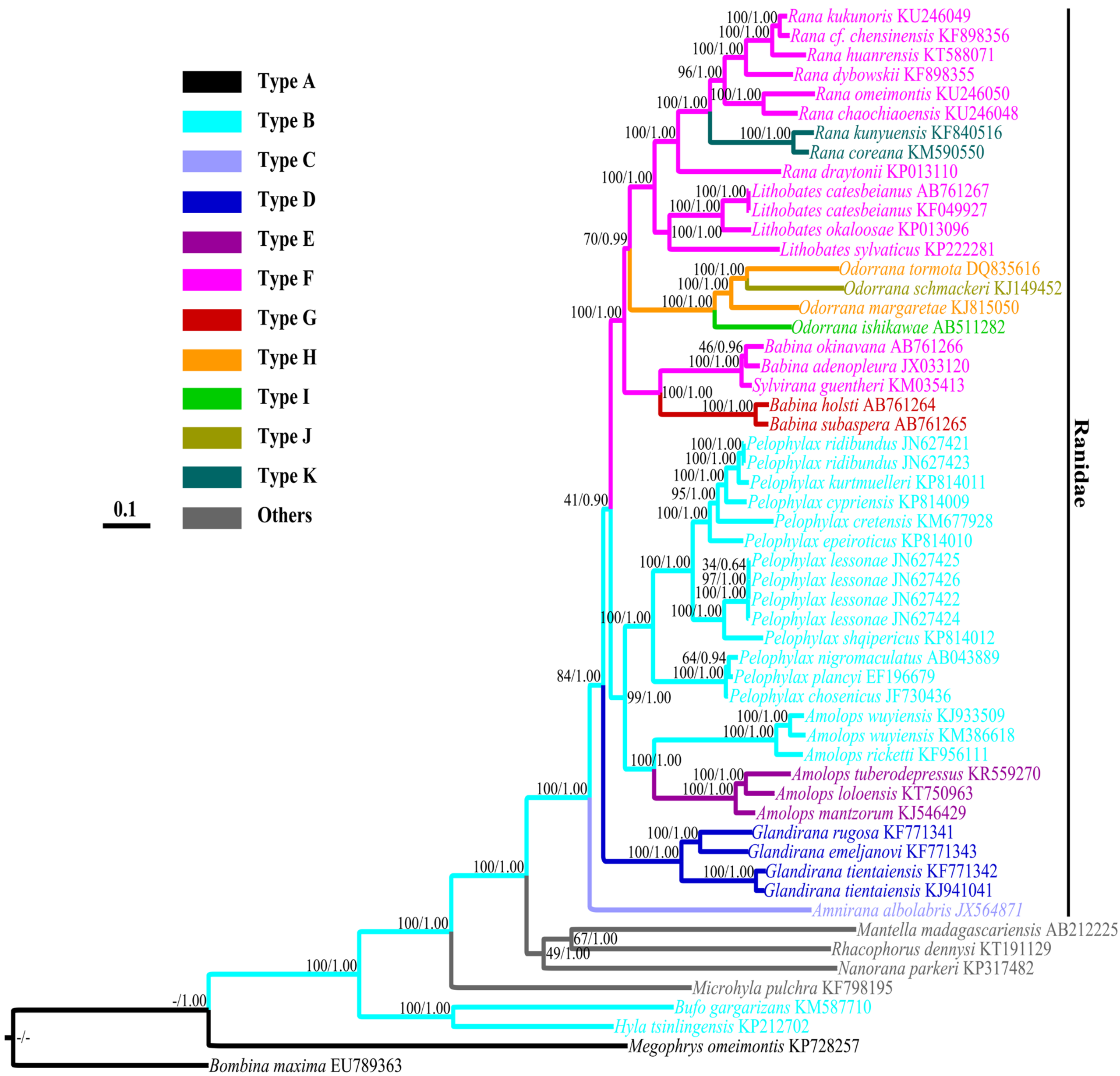


Figure 2 The ML and BI phylogeny trees derived from the concatenated sequences of 13 protein coding genes and two rRNA genes among Ranidae. Numbers above the lines or beside the nodes are rapid bootstrap proportions calculated with 1 000 replicates and Bayesian posterior probabilities, respectively. The different color represents the different genomic rearrangement features of each species.

Table 1 List of taxa used in this study.

Family/Genus	Species	Synonym	Accession number	Voucher	Size (bp)	Sampling locality
Ingroups (Ranidae)						
Amnirana	*Amnirana albolabris*	*Hylarana albolabris*	JX564871	MVZ 234147	15 171†	Unknown
Amolops	*Amolops ricketti*		KF956111/NC_023949	WUSTW01	17 772	Wugong Mountain, Jiangxi, China
	Amolops wuyiensis		KM386618/NC_025591	Unknown	17 797	Unknown
	Amolops wuyiensis		KJ933509	Unknown	17 479	Unknown
	Amolops tuberodepressus		KR559270	CIB-XM3125	18 348†	Jingdong, Yunnan, China
	Amolops mantzorum		KJ546429/NC_024180	Unknown	17 744	Dayi, Sichuan, China
	Amolops loloensis		KT750963	SM-ZDTW-01	18 926	Shimian, Ya'an, Sichuan, China
Babina	*Babina adenopleura*	*Hylarana adenopleura*	JX033120/NC_018771	A-A-WZ001	18 982	Wenzhou, Zhengjiang, China
	Babina holsti		AB761264/NC_022870	Unknown	19 113	Okinawa Island, Japan
	Babina okinavana		AB761266/NC_022872	Unknown	19 959	Iriomote Island, Japan
	Babina subaspera		AB761265/NC_022871	Unknown	18 525	Amami Island, Japan
Glandirana	*Glandirana tientaiensis*	*Rugosa tientaiensis*	KJ941041/NC_025226	Unknown	17 681	Unknown
	Glandirana tientaiensis	*Rugosa tientaiensis*	KF771342	QLY277	17 347†	Ninghai, Zhejiang, China
	Glandirana emeljanovi		KF771343	XM3124	17 294†	Hiroshima, Japan
	Glandirana rugosa		KF771341	CIB IM3	17 426†	Huanren, Liaoning, China
Lithobates	*Lithobates catesbeianus*	*Rana catesbeiana*	AB761267	Unknown	17 682	Amami Island, Japan
	Lithobates catesbeianus	*Rana catesbeiana*	KF049927/NC_022696	JH-NW-2012001	18 241	Jinhua, Zhejiang, China
	Lithobates okaloosae	*Rana okaloosae*	KP013096	LodgeLab Rokaloosae_1	17 504	Unknown
	Lithobates sylvaticus	*Rana sylvatica*	KP222281/NC_027236	Unknown	17 343	Bishop's Mills, Ontario, Canada
Odorrana	*Odorrana ishikawae*	*Rana ishikawae*	AB511282/NC_015305	IABHU 5275	21 020	Amami Island, Japan
	Odorrana tormota	*Amolops tormotus*	DQ835616/NC_009423	AM04005	17 962	Fuxi, Huangshan, Anhui, China
	Odorrana schmackeri		KJ149452/NC_027827	AM13020	18 302	Qimen, Huangshan, Anhui, China
	Odorrana margaretae		KJ815050/NC_024603	HNNU1207003	17 903	China
Pelophylax	*Pelophylax lessonae*	*Rana lessonae*	JN627422	LZ-01	16 263†	Lesny Zakatek, Poland
	Pelophylax lessonae	*Rana esculenta*	JN627424	SP-03	15 790†	Spytkowice, Poland
	Pelophylax lessonae	*Rana ridibundus*	JN627425	SP-04	15 790†	Spytkowice, Poland
	Pelophylax lessonae	*Rana lessonae*	JN627426	ZUR-01	15 790†	Zurawiec, Poland
	Pelophylax ridibundus	*Rana ridibundus*	JN627423	RAFA-02	15 793†	Rafa, Poland
	Pelophylax ridibundus	*Rana ridibundus*	JN627421	GO-01	16 605†	Popowo, Goplo, Poland
	Pelophylax chosenicus	*Rana chosenica*	JF730436/NC_016059	Unknown	18 357	Chungcheongnam-do, South Korea

(Continued Table 1)

Family/Genus	Species	Synonym	Accession number	Voucher	Size (bp)	Sampling locality
	Pelophylax cypriensis		KP814009/NC_026893	GM157-11	18023	Troodos Dam, Cyprus
	Pelophylax epeiroticus		KP814010/NC_026894	MPFC1392	18030	Arta, Greece
	Pelophylax kurtmuelleri		KP814011/NC_026895	MPFC1475	18020	Skala, Greece
	Pelophylax shqipericus		KP814012/NC_026896	GM1013	17366	Virpazar, Montenegro
	Pelophylax cretensis		KM677928/NC_025575	CR03	17829	Kournas village, Greece
	Pelophylax nigromaculatus	*Rana nigromaculata*	AB043889/NC_002805	Unknown	17804	Hiroshim, Japan
	Pelophylax plancyi	*Rana plancyi*	EF196679/NC_009264	Unknown	17822	Unknown
Rana	*Rana dybowskii*		KF898355/NC_023528	Unknown	18864	Amur River basin of Northeast China
	Rana cf. chensinensis		KF898356/NC_023529	Unknown	18808	Yellow River basin of North China
	Rana kunyuensis	*Rana coreana*	KF840516/NC_024548	Unknown	22255	Kunyu Mountain, Shandong, China
	Rana coreana		KM590550	Unknown	15765†	Unknown
	Rana huanrensis		KT588071	y-d20130058	19253	Huanren, Liaoning, China
	Rana draytonii		KP013110	LodgeLab Rdraytonii_1	17805	Unknown
	Rana omeimontis		KU246050	YC-EMLW-01	19934	Yucheng, Ya'an, Sichuan, China
	Rana kukunoris		KU246049	RG-GYLW-01	18863	Zoige, Sichuan, China
	Rana chaochiaoensis		KU246048	SM-ZJLW-01	18591	Shimian, Ya'an, Sichuan, China
Sylvirana	*Sylvirana guentheri*	*Hylarana guentheri*	KM035413 /NC_024748	Unknown	19053	Huang Mount, Anhui, China
Outgroups						
Bombinatoridae	*Bombina maxima*		EU789363/NC_011049	Unknown	18388	Unknown
Bufonidae	*Bufo gargarizans*	*Bufo minshanicus*	KM587710	Unknown	17719	Danba, Sichuan, China
Dicroglossidae	*Nanorana parkeri*		KP317482/NC_026789	Unknown	17837	Dangxiong, Tibet, China
Hylidae	*Hyla tsinlingensis*		KP212702/NC_026524	HTSIN20141129	18295	Unknown
Mantellidae	*Mantella madagascariensis*		AB212225/NC_007888	IABH-6960	22874	Madagascar
Megophryidae	*Megophrys omeimontis*	*Xenophrys omeimontis*	KP728257	MO-HY130601	17013†	Hongya, Meishan, Sichuan, China
Microhylidae	*Microhyla pulchra*		KF798195/NC_024547	Unknown	16744	Dongguan, Guangdong, China
Rhacophoridae	*Rhacophorus dennysi*		KT191129	RDEN20150618	17572	Meilin, Ningguo, Anhui, China

† The mitogenome is near complete.

Table 2 Frequency of each mitochondrial genome rearrangement type in family Ranidae.

Types	No. of genera†	No. of species†	Species name
A	0 (0.00%)	0 (0.00%)	None
B	2 (22.22%)	12 (30.00%)	*Pelophylax ridibundus* JN627421, *P. ridibundus* JN627423, *P. kurtmuelleri* KP814011, *P. cypriensis* KP814009, *P. cretensis* KM677928, *P. epeiroticus* KP814010, *P. lessonae* JN627425, *P. lessonae* JN627426, *P. lessonae* JN627422, *P. lessonae* JN627424, *P. shqipericus* KP814012, *P. nigromaculatus* AB043889, *P. plancyi* EF196679, *P. chosenicus* JF730436, *Amolops wuyiensis* KJ933509, *A. wuyiensis* KM386618, *A. ricketti* KF956111
C	1 (11.11%)	1 (2.50%)	*Amnirana albolabris* JX564871
D	1 (11.11%)	3 (7.50%)	*Glandirana rugosa* KF771341, *G. emeljanovi* KF771343, *G. tientaiensis* KF771342, *G. tientaiensis* KJ941041
E	1 (11.11%)	3 (7.50%)	*Amolops tuberodepressus* KR559270, *A. loloensis* KT750963, *A. mantzorum* KJ546429
F	4 (44.44%)	13 (32.50%)	*Rana kukunoris* KU246049, *R.* cf. *chensinensis* KF898356, *R. huanrensis* KT588071, *R. dybowskii* KF898355, *R. omeimontis* KU246050, *R. chaochiaoensis* KU246048, *R. draytonii* KP013110, *Lithobates catesbeianus* AB761267, *L. catesbeianus* KF049927, *L. sylvaticus* KP222281, *L. okaloosae* KP013096, *Babina okinavana* AB761266, *B. adenopleura* JX033120, *Sylvirana guentheri* KM035413
G	1 (11.11%)	2 (5.00%)	*Babina holsti* AB761264, *B. subaspera* AB761265
H	1 (11.11%)	2 (5.00%)	*Odorrana tormota* DQ835616, *O. margaretae* KJ815050
I	1 (11.11%)	1 (2.50%)	*Odorrana ishikawae* AB511282
J	1 (11.11%)	1 (2.50%)	*Odorrana schmackeri* KJ149452
K	1 (11.11%)	2 (5.00%)	*Rana kunyuensis* KF840516, *R. coreana* KM590550

†The total of genera and species used in this study is 9 and 40, respectively.

Table 3 The mitochondrial genome types in the nine genera of the family Ranidae.

Genera	No. of species	Types
Amnirana	1	C
Amolops	5	B, E
Babina	4	F, G
Glandirana	3	D
Lithobates	3	F
Odorrana	4	H, I, J
Pelophylax	10	B
Rana	9	F, K
Sylvirana	1	F

was quite large, and four *Odorrana* species held the three types (H, I, and J). In all three *Odorrana* rearrangement types, the *trnH* was translocated to D-loop downstream, forming a *HLTPF-trn* cluster. Moreover, the position exchange between *trnN* and O_L was only discovered in Type I (*O. ishikawae*) and Type J (*O. schmackeri*). In particular, the O_L region was triplicate in Type I (*O. ishikawae*). *R. kunyuensis* and *R. coreana* shared the identical arrangement order Type K. Compared with Type F, this type showed more complex variations: one additional D-loop region was inserted into the upstream of *TPF-trn* cluster, and the *ND5* was translocated from the typical $trnS^{AGY}$ downstream to the $trnL^{CUN}$ downstream (Figure 3).

4. Discussion

4.1. Characteristics analysis of the *Rana* mitogenomes Three *Rana* mitogenomes shared the identical genomic organization with those of *R.* cf. *chensinensis*, *R. dybowskii*, *R. huanrensis* and *R. draytonii* (Dong *et al.*, 2016; Li e*t al.*, 2016a; Figure S1), and this genomic organization was similar to the typical Neobatrachian-type (Kurabayashi *et al.*, 2010; Sumida *et al.*, 2001). The variation of molecular size and base composition of entire genome among all published *Rana* mitogenomes were primarily due to the duplication of D-loop region and the variable numbers of tandem repeat element in D-loop region (Dong *et al.*, 2016; Li e*t al.*, 2016a, b). The incomplete termination codon T frequently appeared in seven PCGs, and it was completed by post-transcriptional polyadenylation (Ojala *et al.*, 1981).

4.2. Molecular phylogenetic analysis Overall, the genus level phylogeny reconstructed in our study was congruent with the hypotheses from Li *et al.* (2014) and Bu *et al.* (2016) but conflicted with other results from some



Figure 3 Putative mechanism of gene rearrangement of the mitochondrial genome according to the duplication and random loss model. The information of each gene or region is the same as those in Figure 1. The solid arrows represent duplication events and the dashed arrows represent random loss events. The green and blue boxes represent duplication regions; the gray and black boxes represent partial loss and complete deletion, respectively.

researchers (e.g. Che *et al.*, 2007; Kurabayashi *et al.*, 2010; Ni *et al.*, 2016; Pyron and Wiens, 2011; Wiens *et al.*, 2009; Xia *et al.*, 2014). Our trees placed *Glandirana* at a more basal position with strong support (BP = 84, BPP = 1.00), which was in agreement with the result of Bu *et al.* (2016) but different from other reports that located the *Glandirana* in a nested position within the Ranidae phylogenetic tree with weak statistical support (e.g. Che *et al.*, 2007; Kurabayashi *et al.*, 2010; Ni *et al.*, 2016; Xia *et al.*, 2014). By reviewing the previous work, we found [*Babina*] and [*Lithobates* + *Rana*] had been considered as the sister group of *Odorrana*. Using the single gene or very few genes (e.g. two rRNAs), Che *et al.* (2007), Kurabayashi *et al.* (2010), Wiens *et al.* (2009) and Xia *et al.* (2014) found [*Babina*] was the sister group of *Odorrana*. Kakehashi *et al.* (2013) reconstructed the same phylogenetic relationship using two rRNAs and 13 PCGs and proposed plausible explanation according to the probable gene rearrangement mechanisms (see below).

However, our results robustly supported that [*Lithobates* + *Rana*] was the sister group of *Odorrana*, which was compatible with other studies based on 13 PCGs (e.g. Bu e*t al.*, 2016; Li *et al.*, 2014; Ni *et al.*, 2016; Xue *et al.*, 2016). Kakehashi *et al.* (2013) also noted that the genus *Babina* species formed a monophyletic group (BP = 100). However, the *S. guentheri* was nested in *Babina* clade in our phylogenetic trees, as previously reported by Ni *et al.* (2016). The taxonomic history of *S. guentheri* was somewhat complicated (Wu *et al.*, 2016). From 1882 to 2010, this species was successively placed into several genera, such as *Rana*, *Hylorana*, *Hylarana*, and *Boulengerana* (see Frost, 2017). Most recently, it has been classified into *Sylvirana* based on two mitochondrial and four nuclear gene data (Oliver *et al.*, 2015). Nonetheless, more convincing evidence is indispensable for determining the taxonomic status of this frog.

4.3. Extensive gene rearrangement in Ranidae Kurabayashi *et al.* (2010) stated that the diversity of the mitochondrial genome reorganization in ranids was unexpected. In this study, we summarized 10 different gene orders, and found that all rearrangements occurred at the *ND4–trnF* region and the *trnW–CO1* region. In Caudata mitogenomes, the gene rearrangements also appeared at the two regions (Xia *et al.*, 2010). In Gymnophiona mitogenomes, the gene rearrangements occurred more at the *trnW–CO1* region (San Mauro *et al.*, 2006). Li *et al.* (2010) indicated that the Anura mitogenome rearrangements mainly occurred at the flanks of D-loop region, the margin of O_L structure and the *IQM-trn* genes cluster. Moreover, we found many rearranged patterns, such as *WA*$O_L O_L O_L$*NCY*, *W*O_L*ANCY*, *WN*O_L*CAY* and *WA*O_L*NCY*, are discovered in some Ranidae mitochondrial genomes (Figure 1; Figure 2). Therefore, we speculated the *trnW–CO1* region and the *ND4–trnF* region should be the hotspots of Ranidae mitochondrial genomes rearrangement.

All *Amolops* mitogenomes analyzed in this study were classified as Type B and Type E, and they were different from the previously determined *A. larutensis* rearrangement type (Kurabayashi *et al.*, 2010; Figure 1), implying that the *Amolops* gene rearrangements were various. In particular, the O_L region was triplicate in *O. ishikawae* mitogenome (Type I). The triploidization of the O_L was unique to this frog in Ranidae, but it was also discovered in the mitogenome of *Callulina kreffti* (Brevicipitidae), another Neobatrachia frog (Zhang *et al.*, 2013). In addition, the diploidization of the O_L was found in the *A. larutensis* mitogenome (Kurabayashi *et al.*, 2010).

Interestingly, *R. kunyuensis* and *R. coreana* shared one additional D-loop region and duplicate D-loop regions was not unique to these two ranids (Li *et al.*, 2016b), because it was also discovered in another Ranidae species *A. larutensis* (Kurabayashi *et al.*, 2010), and other Neobatrachia taxa, such as Afrobatrachia frogs (Kurabayashi and Sumida, 2013), Mantellidae frogs (Kurabayashi *et al.*, 2006, 2008), *Rhacophorus schlegelii* (Sano *et al.*, 2005), and *Hoplobatrachus* spp. (Alam *et al.*, 2010; Yu *et al.*, 2012b). Wang *et al.* (2015) found that the duplicated D-loop regions within one individual were almost identical in the bushtits mitochondrial genomes, and further supposed that homologous recombination occurred between paralogous D-loop regions from different mtDNA molecule was proposed as the most suitable mechanism for concerted evolution of the duplicated D-loop regions. Unfortunately, in this study we cannot speculate the mechanism for this *Rana* duplicated D-loop regions.

4.4. Mechanisms and systematic implication of mitochondrial gene rearrangement Generally, the vertebrate mitochondrial gene rearrangement was relatively rare and random (Xia *et al.*, 2014). As stated by many scholars, all observed gene rearrangement events of vertebrate mitogenomes could be classified as translocation, inversion, shuffling, deletion, or duplication (Dowton *et al.*, 2003; Macey *et al.*, 1997), and gene shuffling was the prevailing gene rearrangement type (Macey *et al.*, 1997). In our study, only gene translocation and duplication were discovered in these Ranidae

mitogenomes, and gene shuffling was more common than gene duplication (Figure 1; Figure 3). Unlike the D-loop region and O_L structure, which tend to gene duplication, the tRNAs genes and PCGs tend to gene shuffling (Figure 1; Figure 3). For the formation of rearrangement types, several different rearrangement mechanisms were proposed, such as the tandem duplication and random loss model (Macey *et al.*, 1997; Moritz and Brown, 1987), the tandem duplication and non-random loss model (Lavrov *et al.*, 2002), and the intramitochondrial recombination model (Poulton *et al.*, 1993).

Currently, the duplication and random loss model can be used to explain for most of the animal mitogenome reorganization (e.g. Kakehashi *et al.*, 2013; Kurabayashi *et al.*, 2008). In this model, initially, a duplication including a part of the entire genome happened accidentally because of replication errors (either slipped-strand mispairing or inaccurate termination); then, one of the duplicates of the included genes (or non-coding region) was converted into a pseudogene and subsequently excised from the genome through an accumulation of natural mutations (Dowton *et al.*, 2003; Macey *et al.*, 1997; Moritz and Brown, 1987). In the present study, the duplication and random loss model also could explain all rearrangement events discovered in the Ranidae mitogenomes (Figure 3), although some of our views were not compatible with previous views (e.g. Kakehashi *et al.*, 2013; Kurabayashi *et al.*, 2010). Additionally, it was almost impossible that the same gene order was generated independently through different pathways among different taxa.

The vertebrate mitochondrial rearrangement was regarded as unique, random, and a generally rare event (Boore and Brown, 1998; Liu and Huang, 2010; Xia *et al.*, 2010, 2014), and the occurrence of identical gene rearrangements in two or more lineages indicated that this gene rearrangement type was a synapomorphic type and these lineages were derived from a common ancestor (Macey *et al.*, 1997), although a few convergent or parallel gene rearrangements have been observed in the vertebrate mtDNAs (e.g. Morrison *et al.*, 2002; Wei *et al.*, 2014). The remarkable mitochondrial gene rearrangement contributes to our understanding of phylogenetic relationships and is now considered as a valuable molecular marker (Boore and Brown, 1998; Kurabayashi *et al.*, 2008, 2010; Macey *et al.*, 1997), being widely applied to explore the phylogenetic relationships among various groups at different taxonomic levels (e.g. Kakehashi *et al.*, 2013; Kurabayashi *et al.*, 2006, 2010; Liu *et al.*, 2016; San Mauro *et al.*, 2004, 2014; Wei *et al.*, 2014; Xia *et al.*, 2010; Xue *et al.*, 2016; Zhang *et al.*, 2008, 2009, 2013).

As mentioned above, the previous studies considered [*Babina*] or [*Lithobates* + *Rana*] as the sister taxon of *Odorrana* (e.g. Kakehashi *et al.*, 2013; Kurabayashi *et al.*, 2010; Ni *et al.*, 2016; Xue *et al.*, 2016). Additionally, Kakehashi *et al.* (2013) further pointed out that the [*Babina* + *Odorrana*] clade shared a common ancestral gene arrangement type.

Alternatively, we proposed another explanation: all taxa, incluing *Babina*, *Sylvirana*, *Odorrana*, *Lithobates*, and *Rana*, shared a common ancestral gene order Type F (Figure 2; Figure 3), but this order was completely different from the pattern (*ND4-trnH-trnS$_2$-ND5-ND6-trnE-CYTB*-D-loop-*trnH-trnS$_2$-ND5-ND6-trnE-trnL$_1$-trnT-trnP-trnF-12S-trnV-12S*) inferred by Kakehashi *et al.* (2013). Several lineages possessed their distinctive gene rearrangements, including *Glandirana* spp., *Amolops mantzorum* species group, *Amolops ricketti* species group, *Pelophylax* spp., and the *Rana* + *Lithobates* lineage (excluding *R. kunyuensis* and *R. coreana*). The genus *Amolops* was a complicated group. In sibling *A. larutensis*, a lot of mitochondrial gene rearrangements (including duplication of D-loop region, duplication of O_L, transpositions of *trnK*, *trnH* and *trnG-ND3* block) had been discovered by Kurabayashi *et al.* (2010). Considering the fact that this species possessed a nested position within *Amolops*, Kurabayashi *et al.* (2010) inferred the genomic reorganization was likely to have occurred in a common ancestor of *Amolops*, or during the diversification of this taxon. Now, the latter was confirmed by more available mitogenomes. In *Glandirana* spp., the *trnS^{AGY}* pseudogene was proved as a valuable molecular marker for its phylogenetic analysis.

5. Conclusion

The three *Rana* frogs shared the identical mitogenome arrangement type, which was extremely similar to the typical Neobatrachian-type arrangement shared by most frogs. The phylogenetic analysis using PCGs and rRNAs sequences from 55 mitogenomes demonstrated that the genus *Amnirana* occupied the most basal position among the Ranidae and the [*Lithobates* + *Rana*] was the closest sister group of *Odorrana*. The diversity of Ranidae mitogenome arrangements was unexpected high, and the 47 mitogenomes of 40 ranids were classified into 10 different gene rearrangement types. Some taxa owned their distinctive gene rearrangement characteristics, which had significant implication for their phylogeny

analysis. The tandem duplication and random loss model can explain all rearrangement events discovered in all Ranidae mitogenomes.

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References

Alam M. S., Kurabayashi A., Hayashi Y., Sano N., Khan M. R., Fujii T., Sumida M. 2010. Complete mitochondrial genomes and novel gene rearrangements in two dicroglossid frogs, *Hoplobatrachus tigerinus* and *Euphlyctis hexadactylus*, from Bangladesh. Genes Genet Syst, 85: 219–232

Anderson S., Bankier A. T., Barrell B. G., de Bruijn M. H. L., Coulson A. R., Drouin J., Eperon I. C., Nierlich D. P., Roe B. A., Sanger F., Schreier P. H., Smith A. J. H., Staden R., Young I. G. 1981. Sequence and organization of the mitochondrial human genome. Nature, 290: 457–465

AmphibiaWeb. 2017. Information on amphibian biology and conservation. Retrieved from http://amphibiaweb.org

Boore J. L., Brown W. M. 1998. Big trees from little genomes: mitochondrial gene order as a phylogenetic tool. Curr Opin Genet Dev, 8: 668–674

Bu X., Zhang L., He K., Jiang Y., Nie L. 2016. The complete mitochondrial genome of the *Odorrana schmackeri* (Anura, Ranidae). Mitochondrial DNA Part B, 1: 162–163

Cameron S. L. 2014. How to sequence and annotate insect mitochondrial genomes for systematic and comparative genomics research. Syst Entomol, 39: 400–411

Che J., Pang J., Zhao H., Wu G. F., Zhao E. M., Zhang Y. P. 2007. Phylogeny of Raninae (Anura: Ranidae) inferred from mitochondrial and nuclear sequences. Mol Phylogenet Evol, 43: 1–13

Chen G., Wang B., Liu J., Xie F., Jiang J. 2011. Complete mitochondrial genome of *Nanorana pleskei* (Amphibia: Anura: Dicroglossidae) and evolutionary characteristics of the amphibian mitochondrial genomes. Curr Zool, 57: 785–805

Chen Z., Zhang J., Zhai X., Zhu Y., Chen X. 2015. Complete mitochondrial genome of the green odorous frog *Odorrana margaretae* (Anura: Ranidae). Mitochondrial DNA Part A, 26: 487–488

Darriba D., Taboada G. L., Doallo R., Posada D. 2012. jModelTest 2: More models, new heuristics and parallel computing. Nat Methods, 9: 772

Desjardins P., Morais R. 1990. Sequence and gene organization of the chicken mitochondrial genome: A novel gene order in higher vertebrates. J Mol Biol, 212: 599–634

Dong B., Yu Z., Yang B. 2016. The complete mitochondrial genome of the *Rana huanrensis* (Anura: Ranidae). Mitochondrial DNA Part A, 27: 4551–4552

Dowton M., Castro L. R., Campbell S. L., Bargon S. D., Austin A. D. 2003. Frequent mitochondrial gene rearrangements at the hymenopteran nad3–nad5 junction. J Mol Evol, 56: 517–526

Frost D. R. 2017. Amphibian Species of the World: an Online Reference Version 6.0. Retrieved from http://research.amnh.org/herpetology/amphibia/index.html

Guindon S., Dufayard J. F., Lefort V., Anisimova M., Hordijk W., Gascuel O. 2010. New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. Syst Biol, 59: 307–321

Kakehashi R., Kurabayashi A., Oumi S., Katsuren S., Hoso M., Sumida M. 2013. Mitochondrial genomes of Japanese Babina frogs (Ranidae, Anura): unique gene arrangements and the phylogenetic position of genus *Babina*. Genes Genet Syst, 88: 59–67

Kumazawa Y., Endo H. 2004. Mitochondrial genome of the Komodo dragon: efficient sequencing method with reptile-oriented primers and novel generearrangements. DNA Res, 11: 115-125

Kurabayashi A., Sumida M. 2013. Afrobatrachian mitochondrial genomes: Genome reorganization, gene rearrangement mechanisms, and evolutionary trends of duplicated and rearranged genes. BMC Genomics, 14: 633

Kurabayashi A., Sumida M., Yonekawa H., Glaw F., Vences M., Hasegawa M. 2008. Phylogeny, recombination, and mechanisms of stepwise mitochondrial genome reorganization in mantellid frogs from Madagascar. Mol Biol Evol, 25: 874–891

Kurabayashi A., Usuki C., Mikami N., Fujii T., Yonekawa H., Sumida M., Hasegawa M. 2006. Complete nucleotide sequence of the mitochondrial genome of a Malagasy poison frog *Mantella madagascariensis*: evolutionary implications on mitochondrial genomes of higher anuran groups. Mol Phylogenet Evol, 39: 223–236

Kurabayashi A., Yoshikawa N., Sato N., Hayashi Y., Oumi S., Fujii T., Sumida M. 2010. Complete mitochondrial DNA sequence of the endangered frog *Odorrana ishikawae* (family Ranidae) and unexpected diversity of mt gene arrangements in ranids. Mol Phylogenet Evol, 56: 543–553

Larkin M. A., Blackshields G., Brown N. P., Chenna R., McGettigan P. A., McWilliam H., Valentin F., Wallace I. M., Wilm A., Lopez R., Thompson J. D., Gibson T. J., Higgins D. G. 2007. Clustal W and Clustal X version 2.0. Bioinformatics, 23: 2947–2948

Lavrov D. V., Boore J. L., Brown W. M. 2002. Complete mtDNA sequences of two millipedes suggest a new model for mitochondrial gene rearrangements: duplication and nonrandom loss. Mol Biol Evol, 19: 163–169

Li J., Lei G., Fu C. 2016a. Complete mitochondrial genomes of two brown frogs, *Rana dybowskii* and *Rana* cf. *chensinensis* (Anura: Ranidae). Mitochondrial DNA Part A, 27: 155–156

Li J., Yin W., Xia R., Lei G., Fu C. 2016b. Complete mitochondrial genome of a brown frog, *Rana kunyuensis* (Anura: Ranidae). Mitochondrial DNA Part A, 27: 34–35

Li X. Q., Zhang M., Wang Y. S., Wu X. B. 2010. Research rogress on mitochondrial genomes and gene rearrangements in Anura. In: Ji X. (Ed) Herpetological sinaca 12th edn. Southeast University Press, Nanjing, pp: 387–394

Li Y., Zhang H., Wu X., Xue H., Yan P., Wu X. 2014. A novel mitogenomic rearrangement for *Odorrana schmackeri* (Anura: Ranidae) and phylogeny of Ranidae inferred from thirteen

mitochondrial protein-coding genes. Amphibia-Reptilia, 35: 331–343

Liu J., Xue R., Wang Y., Li D., Yan Q., Yang J. 2016. The near-complete mitogenome sequence of the Omei Horned Toad *Megophrys omeimontis* Liu, 1950 (Anura, Megophryidae). Mitochondrial DNA Part A, 27: 2389–2390

Liu N., Huang Y. 2010. Complete mitochondrial genome sequence of *Acrida Cinerea* (Acrididae: Orthoptera) and comparative analysis of mitochondrial genomes in orthoptera. Int J Genomics, 2010: 319486

Liu Z. Q., Wang Y. Q., Su B. 2005. The mitochondrial genome organization of the rice frog, *Fejervarya limnocharis* (Amphibia: Anura): a new gene order in the vertebrate mtDNA. Gene, 346: 145–151

Luo A., Qiao H., Zhang Y., Shi W., Ho S. Y. W., Xu W., Zhang A., Zhu C. 2010. Performance of criteria for selecting evolutionary models in phylogenetics: a comprehensive study based on simulated datasets. BMC Evol Biol, 10: 242

Macey J. R., Larson A., Ananjeva N. B., Fang Z., Papenfuss T. J. 1997. Two novel gene orders and the role of light-strand replication in rearrangement of the vertebrate mitochondrial genome. Mol Biol Evol, 14: 91–104

Mindell D. P., Sorenson M. D., Dimcheffe D. E. 1998. Multiple independent origins of mitochondrial gene order in birds. Proc Natl Acad Sci USA, 95: 10693–10697

Moritz C., Brown W. M. 1987. Tandem duplications in animal mitochondrial DNAs: variation in incidence and gene content among lizards. Proc Natl Acad Sci USA, 84: 7183–7187

Morrison C. L., Harvey A. W., Lavery S., Tieu K., Huang Y., Cunningham W. C. 2002. Mitochondrial gene rearrangements confirm the parallel evolution of the crab-like form. Proc R Soc Lond B, 269: 345–350

Mueller R. L., Boore J. L. 2005. Molecular mechanisms of extensive mitochondrial gene rearrangement in plethodontid salamanders. Mol Biol Evol, 22: 2104–2112

Ni N., Yu D., Storey K. B., Zheng R., Zhang J. 2016. The complete mitochondrial genome of *Lithobates sylvaticus* (Anura: Ranidae). Mitochondrial DNA Part A, 27: 2460–2461

Ojala D., Montoya J., Attardi G. 1981. tRNA punctuation model of RNA processing in human mitochondria. Nature, 290: 470–474

Oliver L. A., Prendini E., Kraus F., Raxworthy C. J. 2015. Systematics and biogeography of the *Hylarana* frog (Anura: Ranidae) radiation across tropical Australasia, Southeast Asia, and Africa. Mol Phylogenet Evol, 90: 176–192

Pabijan M., Spolsky C., Uzzell T., Szymura J. M. 2008. Comparative analysis of mitochondrial genomes in *Bombina* (Anura; Bombinatoridae). J Mol Evol, 67: 246–256

Poulton J., Deadman M. E., Bindoff L., Morten K., Land J., Brown G. 1993. Families of mtDNA re-arrangements can be detected in patients with mtDNA deletions: duplications may be a transient intermediate form. Hum Mol Genet, 2: 23–30

Pyron R. A., Wiens J. J. 2011. A large-scale phylogeny of Amphibia including over 2800 species, and a revised classification of extant frogs, salamanders, and caecilians. Mol Phylogenet Evol, 61: 543–583

Roe B. A., Ma D. P., Wilson R. K. E., Wong J. F. H. 1985. The complete nucleotide sequence of the *Xenopus laevis* mitochondrial genome. J Biol Chem, 260: 9759–9774

Ronquist F., Huelsenbeck J. P. 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics, 19: 1572–1574

San Mauro D., Gower D. J., Oommen O. V., Wilkinson M., Zardoya R. 2004. Phylogeny of caecilian amphibians (Gymnophiona) based on complete mitochondrial genomes and nuclear RAG1. Mol Phylogenet Evol, 33: 413–427

San Mauro D., Gower D. J., Zardoya R., Wilkinson M. 2006. A hotspot of gene order rearrangement by tandem duplication and random loss in the vertebrate mitochondrial genome. Mol Biol Evol, 23: 227–234

San Mauro D., Gower D. J., Müller H., Loader S. P., Zardoya R., Nussbaum R. A., Wilkinson M. 2014. Life-history evolution and mitogenomic phylogeny of caecilian amphibians. Mol Phylogenet Evol, 73: 177–189

Sano N., Kurabayashi A., Fujii T., Yonekawa H., Sumida M. 2005. Complete nucleotide sequence of the mitochondrial genome of Schlegel's tree frog *Rhacophorus schlegelii* (family Rhacophoridae): duplicated control regions and gene rearrangements. Genes Genet Syst, 80: 213–224

Su X., Wu X. B., Yan P., Cao S. Y., Hu Y. L. 2007. Rearrangement of a mitochondrial tRNA gene of the concave-eared torrent frog, *Amolops tormotus*. Gene, 394: 25–34

Sumida M., Kanamori Y., Kaneda H., Kato Y., Nishioka M., Hasegawa M., Yonekawa H. 2001. Complete nucleotide sequence and gene rearrangement of the mitochondrial genome of the Japanese pond frog *Rana nigromaculata*. Genes Genet Syst, 76: 311–325

Sumida M., KondoY., Kanamori Y., Nishioka M. 2002. Inter- and intraspecific evolutionary relationships of the rice frog *Rana limnocharis* and the allied species *R. cancrivora* inferred from crossing experiments and mitochondrial DNA sequences of the 12S and 16S rRNA genes. Mol Phylogenet Evol, 25: 293–305

Tamura K., Stecher G., Peterson D., Filipski A., Kumar S. 2013. MEGA6: molecular evolutionary genetics analysis version 6.0. Mol Biol Evol, 30: 2725–2729

Tzeng C. S., Hui C. F., Shen S. C., Huang P. C. 1992. The complete nucleotide sequence of the *Crossostoma lacustre* mitochondrial genome: conservation and variations among vertebrates. Nucl Acids Res, 20: 4853–4858

Wang X., Huang Y., Liu N., Yang J., Lei F. 2015. Seven complete mitochondrial genome sequences of bushtits (Passeriformes, Aegithalidae, *Aegithalos*): the evolution pattern in duplicated control regions. Mitochondrial DNA, 26: 1–7

Wei S. J., Li Q., Achterberg K. V., Chen X. X. 2014. Two mitochondrial genomes from the families Bethylidae and Mutillidae: independent rearrangement of protein-coding genes and higher-level phylogeny of the Hymenoptera. Mol Phylogenet Evol, 77: 1–10

Wiens J. J., Sukumaran J., Pyron R. A., Brown R. M. 2009. Evolutionary and biogeographic origins of high tropical diversity in Old World frogs (Ranidae). Evolution, 63: 1217–1231

Wu X., Li Y., Zhang H., Jiang Z., Xue H., Yan P., Wu X. 2016. The complete mitochondrial genome of *Hylarana guentheri* (Amphidia, Anura, Ranidae). Mitochondrial DNA Part A, 27: 1223–1224

Xia Y., Peng R., Zeng X. M. 2010. Characteristics of mitochondrial

gene rearrangement in Caudata. In: Ji X. (ed) Herpetological sinaca 12th edn. Southeast University Press, Nanjing, pp: 363–371

Xia Y., Zheng Y., Miura I., Wong P. B., Murphy R. W., Zeng X. 2014. The evolution of mitochondrial genomes in modern frogs (Neobatrachia): nonadaptive evolution of mitochondrial genome reorganization. BMC Genomics, 15: 691

Xue R., Liu J., Yu J., Yang J. 2016. The complete mitogenome of *Amolops loloensis* and related phylogenetic relationship among Ranidae. Mitochondrial DNA Part A, 27: 4629–4630

Yan L., Geng Z. Z., Yan P., Wu X. B. 2016. The complete mitochondrial genome of *Glandirana tientaiensis* (Ranidae, Anura). Mitochondrial DNA Part A, 27: 1154–1155

Yu D., Zhang J., Zheng R. 2012a. The complete mitochondrial genome of *Babina adenopleura* (Anura: Ranidae). Mitochondrial DNA Part A, 23: 423–425

Yu D., Zhang J., Zheng R., Shao C. 2012b. The complete mitochondrial genome of *Hoplobatrachus rugulosus* (Anura: Dicroglossidae). Mitochondrial DNA Part A, 23: 336–337

Zardoya R., Garrido-Pertierra A., Bautista J. M. 1995. The complete nucleotide sequence of the mitochondrial DNA genome of the rainbow trout, *Oncorhynchus mykiss*. J Mol Evol, 41: 942–951

Zhang P., Papenfuss T. J., Wake M. H., Qu L., Wake D. B. 2008. Phylogeny and biogeography of the family Salamandridae (Amphibia: Caudata) inferred from complete mitochondrial genomes. Mol Phylogenet Evol, 49: 586–597

Zhang P., Wake M. H. 2009. A mitogenomic perspective on the phylogeny and biogeography of living caecilians (Amphibia: Gymnophiona). Mol Phylogenet Evol, 53: 479–491

Zhang P., Liang D., Mao R. L., Hillis D. M., Wake D. B., Cannatella D. C. 2013. Efficient sequencing of anuran mtDNAs and a mitogenomic exploration of the phylogeny and evolution of frogs. Mol Biol Evol, 30: 1899–1915

Appendix

Table S1 Details of the primers used to amplify the entire mitogenomes of the three *Rana* species.

No.	Name	*Rana omeimontis*		*Rana chaochiaoensis*		*Rana kukunoris*		Sequences (5' end–3' end)[†]	Source
		Location	Length	Location	Length	Location	Length		
1	FSO1	250	5 290	250	5 364	250	5 279	AACGCTAAGATGAACCCTAA AAAGTTCT	Kumazawa *et al*., 2004
	ND1daH	5 539		5 613		5 528		AAAATCAGCGGTRAATATCAC	Kumazawa *et al*., 2004
2	RAsnF	5 336	1 901	5 330	1 981	5 325	1 901	TATCCAGCGAGCTTCATT	Li 2014, unpubl. data
	RAspR1	7 236		7 310		7 225		GTCTTGGAAGCCGAGTTG	Li 2014, unpubl. data
3	RSer1F1	7 067	1 265	7 141	1 265	7 056	1 265	AAAGGAGGGAATTGAACC	Li 2014, unpubl. data
	RATP6R3	8 331		8 405		8 320		AAGAAGGCTCATTTGTGG	Li 2014, unpubl. data
4	RLysF1	7 903	1 719	7 977	1 719	7 892	1 719	TGTAGGTTAGCGACAGCC	Li 2014, unpubl. data
	RGlyR1	96 21		9 695		9 610		GGTGATTGGAAGTCATCTGT	Li 2014, unpubl. data
5	EM4-F	9 188	2 023	-	-	-	-	CCTCCTTAATACAGCCGTTC	This study
	EM4-R	11 210		-		-		TGGCTAACTGAAGATATAGCAA	This study
6	RCO3F5	-	-	9 350	2 050	9 265	2 049	CTTCAAGCCCTTACTATTACA	Li 2014, unpubl. data
	RND4R2	-		11 399		11 313		GTTGGCAAGGCAGAAGAG	Li 2014, unpubl. data
7	RND4F1	10 747	1 061	10 821	1 062	10 735	1 062	CAAGAACGACGMCTWGAAG	Li 2014, unpubl. data
	RND5R2	11 807		11 882		11 796		GYGGTGAGGAATTAGCAG	Li 2014, unpubl. data
8	Rch67F	11 334	655	11 408	656	11 322	646	TGAGCGTACAAATAGCCGAAC	This study
	Rch67R	11 988		12 063		11 967		AGTGAAATAAAGAATGCCGTT	This study
9	RND4F6	11 714	2 969	11 789	2 493	11 703	2 654	AAAAACAYTAGATTGTGATTC	Li 2014, unpubl. data
	RND6R4	14682		14 281		14 356		TATTAKTRGGACTTTTGG	Li 2014, unpubl. data
10	RND6F1	14 500	704	14 099	704	14 174	704	ASGCAGCACARTAAGCAA	Li 2014, unpubl. data
	RcytbR3	15 203		14 802		14 877		CGCCTCARAAGGAYATTTG	Li 2014, unpubl. data
11	EM1-F	15 011	3 093	-	-	-	-	CTTCGTAACCTCCACGCTA	This study
	EM1-R	18 103		-		-		CTTAAAGAGACACTTGCACCA	This study
12	ZJ1-F	-	-	14 679	2 207	-	-	CTCTATTACGGCTCATACCTC	This study
	ZJ1-R	-		16 885		-		TCGGTAATCAAGATAAGTCCA	This study
13	GY2-F	-	-	-	-	14 559	2 122	CTAGGCGTATGTCTTATTGCTC	This study
	GY2-R	-		-		16 680		CGTGTGTTCGATCAACCAA	This study
14	XRACRSF5								
15	EM2-F	17 908	2 743	-	2 613	-	2 667	TTATCGACTACTCCGTGCAT	This study
	ZJ2-F	-		16694		-		ATAAGCCAGTCCTTAATCCTG	This study
	GY1-F	-		-		16 912		ATCTTCATTATTCAAATGGCT	This study
	12S_430Rev	716		715		715		GGGTATCTAATCCCAGTTTG	Sumida *et al*., 2002

[†]Y = C/T, R = A/G, W = A/T, M = A/C, K = G/T, S = G/C

Table S2 Location of features in the three *Rana* mitogenomes.

Gene	Strand	*Rana omeimontis*				*Rana chaochiaoensis*				*Rana kukunoris*			
		Location	Size	Spacer	Codon	Location	Size	Spacer	Codon	Location	Size	Spacer	Codon
tRNA-Leu	H	1..72	73	–		1..72	73	–		1..72	73	–	
tRNA-Thr	H	75..144	70	+2		75..144	70	+2		75..144	70	+2	
tRNA-Pro	L	145..213	69	+0		145..213	69	+0		145..213	69	+0	
tRNA-Phe	H	215..284	70	+1		215..284	70	+1		215..284	70	+1	
12S	H	285..1215	931	+0		285..1214	930	+0		285..1213	929	+0	
tRNA-Val	H	1216..1284	69	+0		1215..1283	69	+0		1214..1282	69	+0	
16S	H	1285..2866	1582	+0		1284..2859	1576	+0		1283..2858	1576	+0	
tRNA-Leu	H	2867..2939	73	+0		2860..2932	73	+0		2859..2931	73	+0	
ND1	H	2979..3900	922	+39	ATC/T--	2993..3893	901	+60	GTG/T--	2932..3889	958	+0	GTG/T--
tRNA-Ile	H	3901..3971	71	+0		3894..3964	71	+0		3890..3960	71	+0	
tRNA-Gly	L	3972..4042	71	+0		3966..4036	71	+1		3961..4031	71	+0	
tRNA-Met	H	4042..4110	69	–1		4036..4104	69	–1		4031..4099	69	–1	
ND2	H	4111..5143	1033	+0	ATG/T--	4105..5137	1033	+0	ATG/T--	4100..5132	1033	+0	ATG/T--
tRNA-Trp	H	5144..5213	70	+0		5138..5207	70	+0		5133..5202	70	+0	
tRNA-Ala	L	5214..5283	70	+0		5208..5277	70	+0		5203..5272	70	+0	
tRNA-Asn	L	5284..5356	73	+0		5278..5350	73	+0		5273..5345	73	+0	
O_L		5357..5387	31	+0		5351..5379	29	+0		5346..5376	31	+0	
tRNA-Cys	L	5385..5449	65	–3		5377..5441	65	–3		5374..5438	65	–3	
tRNA-Tyr	L	5450..5516	67	+0		5442..5508	67	+0		5439..5505	67	+0	
COX1	H	5518..7071	1554	+1	GTG/AGG	5592..7145	1554	+83	GTG/AGG	5507..7060	1554	+1	GTG/AGG
tRNA-Ser	L	7063..7133	71	–9		7137..7207	71	–9		7052..7122	71	–9	
tRNA-Asp	H	7135..7203	69	+1		7209..7277	69	+1		7124..7192	69	+1	
COX2	H	7204..7891	688	+0	ATG/T--	7278..7965	688	+0	ATG/T--	7193..7880	688	+0	ATG/T--
tRNA-Lys	H	7892..7960	69	+0		7966..8034	69	+0		7881..7949	69	+0	
ATP8	H	7962..8123	162	+1	ATG/TAA	8036..8197	162	+1	ATG/TAA	7951..8112	162	+1	ATG/TAA
ATP6	H	8105..8798	694	–19	GTG/T--	8179..8872	694	–19	GTG/T--	8094..8787	694	–19	GTG/T--
COX3	H	8799..9582	784	+0	ATG/T--	8873..9656	784	+0	ATG/T--	8788..9571	784	+0	ATG/T--
tRNA-Gly	H	9583..9650	68	+0		9657..9724	68	+0		9572..9639	68	+0	
ND3	H	9651..9990	340	+0	ATG/T--	9725..10064	340	+0	ATG/T--	9640..9979	340	+0	ATG/T--
tRNA-Arg	H	9991..10060	70	+0		10065..10134	70	+0		9980..10048	69	+0	

(Continued Table S1)

Gene	Strand	*Rana omeimontis*				*Rana chaochiaoensis*				*Rana kukunoris*			
		Location	Size	Spacer	Codon	Location	Size	Spacer	Codon	Location	Size	Spacer	Codon
ND4L	H	10061..10345	285	+0	GTG/TAA	10135..10419	285	+0	GTG/TAA	10049..10333	285	+0	GTG/TAA
ND4	H	10339..11698	1360	–7	ATG/T--	10413..11772	1360	–7	ATG/T--	10327..11686	1360	–7	ATG/T--
tRNA-His	H	11699..11766	68	+0		11773..11841	69	+0		11687..11755	69	+0	
tRNA-Ser	H	11767..11833	67	+0		11842..11908	67	+0		11756..11822	67	+0	
ND5	H	11855..13651	1797	+21	ATG/AGA	11939..13726	1788	+30	ATC/AGA	11825..13630	1806	+2	ATC/AGG
ND6	L	14207..14701	495	+555	ATG/AGA	13806..14300	495	+79	ATG/AGA	13881..14375	495	+50	ATG/AGA
tRNA-Glu	L	14702..14770	69	+0		14301..14369	69	+0		14376..14444	69	+0	
Cytb	H	14774..15916	1143	+3	ATG/TAA	14373..15515	1143	+3	ATG/TAA	14448..15590	1143	+3	ATG/TAA
D-loop		15917..19934	4018	+0		15516..18591	3076	+0		15591..18863	3274	+0	

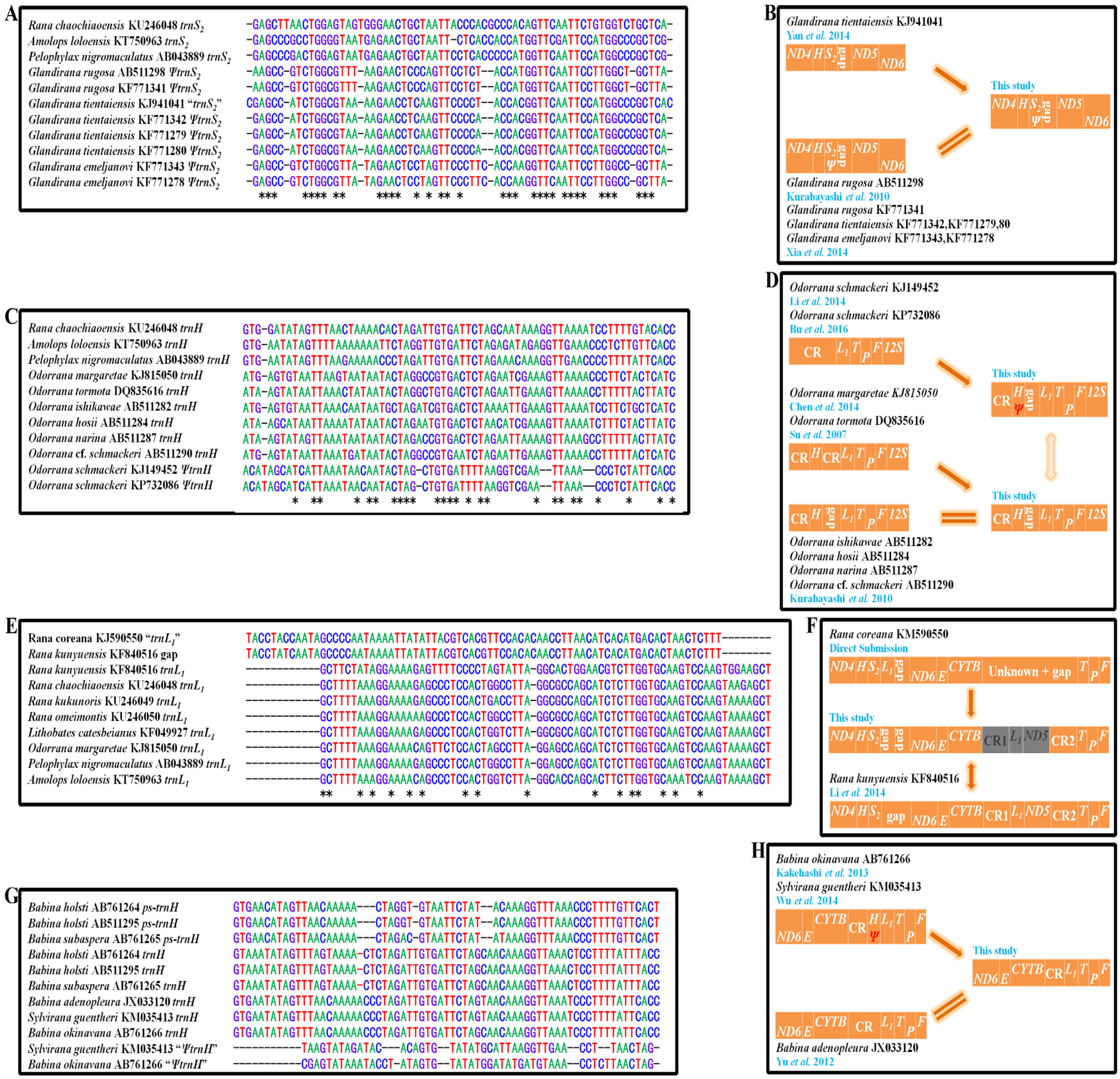


Figure S1 Correction of some mitogenome annotation errors previously submitted to GenBank. (A)–(H) are the homologous sequence alignments and the annotation corrections of the *trnS*^{AGY} gene of *Glandirana* genus, the *trnH* gene of *Odorrana* genus, the *trnL*^{CUN} gene of *Rana* genus, and the *trnH* gene of *Babina* genus, respectively. The information of each gene or region is the same as those in Figure 1, and the ps- and Ψ indicate the corresponding pseudogene. The double quotation marks (e.g. "*trnS*$_2$", "*trnL*$_1$", and "*ps-trnH*") indicate that this annotation is an error.

During the early analysis, we observed that there were many errors in some mitogenome annotations submitted to GenBank. In order to avoid interferences caused by these errors in our subsequent analysis, we reanalyzed all sequences using the online services MitoAnnotator and ARWEN. Overall, we made the following important corrections:

(1) There were four *Glandirana* mitogenomes in our data (Table 1). Yan *et al.*, (2016) reported that the *G. tientaiensis* mitogenome (KJ941041) possessed all 22 tRNAs. However, we were ffailed to detect the presence of *trnS*^{AGY} using the online programs. Additionally, homologous sequences alignments indicated that the primary sequence of the typical location of *trnS*^{AGY} in this mitogenome was very similar to those in the other Ranidae species (Figure S1A). As many scholars reported in *Glandirana* mitogenomes (AB511298, KF771278–80, KF771341–3), the typical locations of *trnS*^{AGY} were 62–63 bp non-coding fragments, which should be identified as pseudogenes (*ps-trnS*^{AGY}, or *Ψ trnS*^{AGY}, Figure S1B) (Kurabayashi *et al.*, 2010; Xia *et al.*, 2014).

(2) Typically, the *trnH* gene was located between the *ND4* and the *trnS*^{AGY} genes in most anuran mitogenomes, but it was translocated in *Odorrana* mitogenomes (Kurabayashi *et al.*, 2010). However, Li *et al.* (2014) reported that the loss of *trnH* was the distinctive feature in the *O. schmackeri* mitogenome (KJ149452). Our homologous sequences alignments confirmed that the primary sequence of the typical location of *trnH* in this mitogenome was very similar to those in other Ranidae mitogenomes and the anticodon really existed (Figure S1C). Therefore, we speculated that the *trnH* in *O. schmackeri* mitogenome was just converted into a pseudogene (*ps-trnH*, or *Ψ trnH*, Figure S1D).

More interestingly, Chen *et al.* (2015) thought that the *trnH* gene was translocated into the D-loop region in *O. margaretae* mitogenome (KJ815050, Figure S1D), as previously reported in *O. tormota* (DQ835616, Figure S1D) (Su *et al.*, 2007). However, our re-annotations of those two genomes were inconsistent with their views but consistent with those of Kurabayashi *et al.* (2010) (Figure S1D). For these reasons, we corrected the organizational order in *Odorrana* mitogenomes as "CR-*trnH* (or *Ψ trnH*)-gap-$trnL^{CUN}$" (Figure S1D).

(3) The GenBank entry KM590550 is a near-complete mitogenome sequence of *R. coreana*. Regrettably, the *tRNA-Phe* gene was abbreviated incorrectly as *trnP* in the original annotation, but in fact it should be *trnF*. Moreover, the putative $trnL^{CUN}$ gene was not present in our prediction and its nucleotide similarity with the corresponding gene in other ranid frogs was very low. Instead, it shared extremely high sequence identity (99%) with the non-coding region between the $trnS^{AGY}$ and *ND6* genes of *R. kunyuensis* mitogenome (Figure S1E). Additionally, *R. kunyuensis* mitogenome owned a novel gene order arrangement with duplicate D-loop regions and a translocation of $trnL^{CUN}$ and *ND5* in comparison with congeneric mitogenomes (Li *et al.*, 2016b). We believed that the real $trnL^{CUN}$ gene should be located at the undetermined part of this mitogenome (KM590550), and these two mitogenomes might share the same gene order arrangement (Figure S1F).

(4) Kurabayashi *et al.* (2010), Kakehashi *et al.* (2013) and Wu *et al.* (2016) successively pointed out that the *Babina* species (*B. holsti* AB511295 and AB761264, *B. subaspera* AB761265 and *B. okinavana* AB761266) and *Sylvirana guentheri* (KM035413) owned one pseudogene of *trnH* (*ps-trnH*, or *Ψ trnH*) located at the downstream position of D-loop region. However, in the sibling *B. adenopleura* mitogenome (JX033120), a similar phenomenon did not occur (Yu *et al.*, 2012a). Here, the multiple sequences alignments showed that the putative *ps-trnH* nucleotide similarities with the real *trnH* in both *B. okinavana* (AB761266) and *S. guentheri* (KM035413) were quite low (Figure S1G). Given that the three species (including *B. okinavana*, *B. adenopleura* and *S. guentheri*) were clustered into a clade in the phylogenetic tree (Figure 2), which to some extent implying that they might share a identical gene order, we inferred the putative *ps-trnH*s were just a segment of the D-loop regions (Figure S1H).

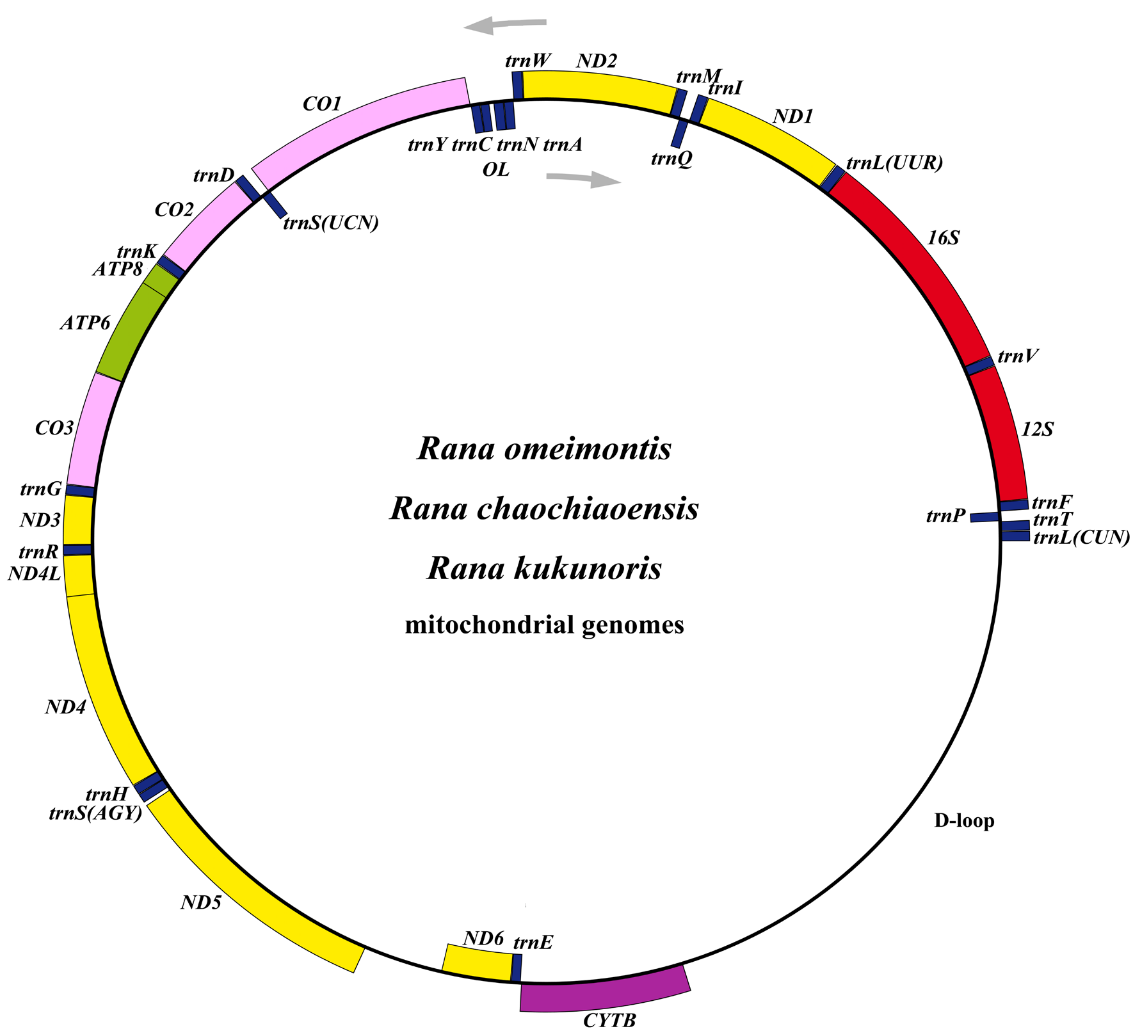


Figure S2 Circular map of the mitochondrial genomes of three *Rana* frog analyzed in this study. Each transfer RNA gene is represented by the standard one-letter amino acid code, and other genes are abbreviated as follows: *12S* and *16S*, 12S and 16S ribosomal RNA; *ATP6* and *ATP8*, adenine triphosphatase subunits 6 and 8; *CO1–3*, cytochrome c oxidase subunits 1–3; *CYTB*, cytochrome b; *ND1–6* and *4L*, NADH dehydrogenase subunits 1–6 and 4L. O_L denotes the replication origin of light strand. Those genes encoded by the heavy and light strands and their respective transcriptional directions are shown outside and inside the circle, respectively.